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Carol Ward [a,b,*] James Meehan [b,c] Mark Gray [a,b] Ian H Kunkler [b] Simon P Langdon [b] Alan Murray [d] David Argyle [a]

[a] The Royal (Dick) School of Veterinary Studies and Roslin Institute, University of Edinburgh, Easter Bush, Roslin, Midlothian, Edinburgh, EH25 9RG, United Kingdom.

[b] Cancer Research UK Edinburgh Centre and Division of Pathology Laboratories, Institute of Genetics and Molecular Medicine, University of Edinburgh, Crewe Road South, Edinburgh, EH4 2XU, United Kingdom.

[c] Institute of Sensors, Signals and Systems, School of Engineering and Physical Sciences, Heriot-Watt University, Edinburgh, EH14 4AS, United Kingdom.

[d] School of Engineering, Faraday Building, The King's Buildings, Mayfield Road, Edinburgh, EH9 3JL, United Kingdom.

*Corresponding author. Tel.: +44 131 5371763.
E-mail address: C.Ward@ed.ac.uk

James Meehan	j.meehan@hw.ac.uk	Tel.: +44 131 5371763.
Mark Gray	s9900757@sms.ed.ac.uk	Tel.: +44 131 5371763.
Ian Kunkler	iankunkler@yahoo.com	Tel.: +44 131 651 8606
Simon Langdon	simon.langdon@ed.ac.uk	Tel.: +44 131 5371763.
David Argyle	david.argyle@roslin.ed.ac.uk	Tel.: +44 131 650 6241
Alan Murray	Alan.Murray@ed.ac.uk	Tel.:+44 131 6505589

Abstract

The immense costs in both financial terms and pre-clinical research effort that occur in the development of anti-cancer drugs are unfortunately not matched by a substantial increase in improved clinical therapies due to the high rate of failure during clinical trials. This may be due to issues with toxicity, or lack of clinical effectiveness when the drug is evaluated in patients. Currently, much cancer research is driven by the need to develop therapies that can exploit cancer cell adaptations to conditions in the tumor microenvironment such as acidosis and hypoxia, the requirement for more-specific, targeted treatments, or the exploitation of 'precision medicine' that can target known genomic changes in patient DNA. The high attrition rate for novel anti-cancer therapies suggests that the pre-clinical methods used in screening anti-cancer drugs need improvement. This chapter considers the advantages and disadvantages of 3D organotypic models in both cancer research and cancer drug screening, particularly in the areas of targeted drugs and the exploitation of genomic changes that can be used for therapeutic advantage in precision medicine.

Introduction

The simple definition of "organoid" is "resembling an organ", implying that it should contain more than one cell type that is typically found in the organ of its origin, demonstrate an organ specific function, and that the cells should show some degree of organization. However, given that in this case the "organ" being modelled is a tumor, most, if not all 3D models meet these criteria. 3D culture can encompass various types of model such as spheroids and organoids, (terms which are often used interchangeably), or explants. Arguably, xenografts are also a type of 3D culture that fits the tumor organoid definition. For sake of clarity, the various 3D systems meeting these criteria will be discussed here separately. For more information on the definition and historical perspective of organoid development, see Simian and Bissell 2017.

Although extensive funding supports cancer research each year, the majority of novel drugs and therapies fail to translate into clinical practice due to lack of success in clinical trials, either because of deficiencies in clinical efficacy, or issues with toxicity (Petsko, 2010; Arrowsmith, 2011). Approximately one drug in 5,000 - 10,000 tested will gain FDA approval, with attrition rates in oncology particularly high with only 5% of anti-cancer therapies successfully undergoing Phase I/II clinical trials (Zamboni et al. 2012, Ocana et al. 2011). This is despite strong and significant *in vitro* and *in vivo* preclinical data, suggesting that better pre-clinical methodologies are required to identify new therapeutics (Kamb, 2005; Caponigro et al. 2011; Singh and Ferrara 2012; Siolas and Hannon 2013). The assessment of novel effective anti-cancer compounds is a compromise between the use of high throughput, low cost testing strategies, and high cost, low throughput methods which may give better translational results.

Most preclinical studies into the efficacy of anti-cancer drugs use cancer cell lines to assess various parameters such as cell survival, proliferation, cell death, migration and invasion. 2D cultures are one of the most cost effective strategies for high throughput assays and are used to test large numbers of compounds and select those for further examination. These cell lines can be exploited to investigate the role of specific pathways, genes or molecules using technologies such as CRISPR

(Sander and Joung 2014; Ledford 2015), siRNA (Moore et al. 2010), or inducible transgenics (Pajic et al. 2000; Belteki et al. 2005; Saunders 2011; Xie et al. 2014). However, these systems lack the heterogeneity of the original tumor, since cell culture selects those clones that best proliferate and survive in culture conditions (Daniel et al. 2009; Hensley et al. 2016). Using a panel of cell lines of the cancer of interest, or from different tumors such as the NCI-60 panel, or the *de novo* expansion of cell lines from tumor tissue, can partially overcome this problem (Burdall et al. 2003; Langdon 2004); but 2D culture cannot replicate the complex stromal elements of tumors, interactions with immune cells, inflammatory mediators, growth factors or the acidic/hypoxic conditions found in the tumor microenvironment (TME) (Karar and Maity 2009; Hanahan and Weinberg 2011; Ward et al. 2013). Targeting the adaptations cancer cells adopt to survive in the tumor microenvironment, is currently a novel approach to the treatment of solid tumors, and more appropriate models are required to support this research (McAllister and Weinberg 2010; Pettersen et al. 2015).

3D *in vivo* models

a) Mouse xenografts

Following assessment in 2D model systems, candidate compounds move into *in vivo* studies: generally mouse xenografts growing human tumor cell lines in strains of immunocompromised mice. These have shown mixed efficacy in translating novel drugs into clinical use, and often reproducibility of results is poor (Sausville and Burger 2006; Boedigheimer et al. 2013); but they do allow *in vivo* monitoring of tumor growth, drug toxicity, efficacy and pharmacokinetic studies, and will reflect the oxygen and pH gradients found in solid tumors. However, some cell lines do not form xenografts, metastatic models are limited, and immune responses, which affect tumor growth are compromised. Although xenografts incorporate some stromal elements, they are murine rather than human (Daniel et al. 2009). Xenografts are selected for rapidity of growth, while human tumors can take years to progress clinically; therefore these models do not replicate the heterogeneity of

the original tumor. Many xenografts are implanted subcutaneously and therefore do not reproduce the specific microenvironment of the tumor under investigation (Polilti and Pao 2011). Orthotopic xenografts enable tumor development in the organ of origin, this can allow evaluation in a preclinical tumor model which more closely mimics the disease process in humans. However these models can be more difficult to establish compared with subcutaneous implantation.

b) Genetically engineered mouse models (GEMMs)

Spontaneous or carcinogen generated tumors, reflect the stromal interactions and angiogenesis found with tumor progression, but in a murine system with functioning immune responses. In GEMMs, tumors originate in a group of cells as a consequence of germline or somatic mutations, and share the genetic heterogeneity and histopathology of human tumors. They allow expression of oncogenes in a tissue-specific manner as well as conditional expression or deletion of oncogenes and tumor suppressors, allowing many cancers to be modelled (Sharpless and Depinho 2006; Politi and Pao 2011). GEMMs are also useful to model treatment resistance, chemotherapy enhancement, co-treatments and biomarker validation (Pietras and Hanahan 2005; Bell-McGuinn et al. 2007; Faca et al. 2008; Pitteri et al. 2009; Bellmunt et al. 2010; Polti et al. 2010). Several studies show that GEMMs model both the TME and stroma more accurately than xenografts (Olive et al. 2009; Graves et al. 2010), and better replicate the sensitivity of cancer to systemic therapy in clinical trials (Singh et al. 2010; Chen et al. 2012). However, the model is still murine.

c) Patient-derived xenografts (PDXs)

PDXs, involving the transfer of patient-derived tumor tissue into immunocompromised mice, (Kopetz et al. 2012; Julien et al. 2012; Huang et al. 2014), show pathology, growth and metastatic outcomes that closely correspond to the original tumor, and sustain the original histological markers, gene expression profiles, and proliferation indices (DeRose et al. 2011; Dean et al. 2012; Tentler et al. 2012; Stewart et al. 2017). This means that experimental data can be complemented with

clinical information, such as response to treatment in the original patient. The use of PDXs is increasing in preclinical studies (Hidalgo et al. 2011; Tentler et al. 2012), in conjunction with 'co-clinical trials' which run preclinical PDX and clinical trials at the same time (Nardella et al. 2011). For example, one study demonstrated that PDXs developed from individual patient tumors could be used to select treatment options with an 88% response rate (Hidalgo et al. 2011). PDXs have also been used to model human phase II trials that can examine differences in therapy responses across different cancers (Townsend et al. 2016; Byrne et al. 2017). PDX models have now superseded the NCI-60 panel as the gold standard for cancer research because of the preservation of heterogeneity (Leford, 2016).

Whilst this system is more robust, PDXs do have some shortcomings. Passaging is time consuming and some human stromal tumor elements are progressively lost over time. The rate of engraftment is poor for some cancer types such as prostate and ER^+ breast cancer (DeRose et al. 2011; Lawrence et al. 2013) and tissue from needle biopsies is often inadequate for growth. There is still deficiency in immune responses and increased growth rate of tumors compared with human cancers (Rayal et al. 2012; Huang et al. 2014), although tumor growth and drug validation in these models can take several months (Hildalgo et al. 2011; Gao and Chen 2015). The same tumor sample can produce PDXs which may differ in genetics, or cell-surface markers, depending on the mouse strains used for tumor growth (Klco et al. 2014). The ratio of cancer cells to stromal cells can also drift; for example, pancreatic ductal adenocarcinomas (PDAs), contain high numbers of stromal cells (Guillaumond et al. 2013; Beloribi-Djefaflia et al. 2015; Delitto et al. 2015), but because cancer cells multiply at a greater rate than stromal cells, stromal/cancer cell interactions change as cell ratios change (Martinez-Garcia et al. 2014). A recent study of 1,100 PDX models across 24 types of cancer showed sizable phenotypic and genomic changes between the progression of PDX models and human cancers, with PDXs developing both chromosome and gene copy number alterations during passages (Ben-David et al. 2017), which could influence the response to drug treatment. Metabolic reprograming has been identified as a key hallmark of cancer

(Hanahan and Weinberg 2011), leading to increased interest in targeting specific enzymes as a novel therapeutic strategy in this disease. However, studies using human lung cancer tissue, show that both glycolysis and glutaminolysis are increased when human lung tumor tissue is cultured in PDX models (with murine stroma) when compared with the same tissue cultured with human stroma (using tissue slices), implying that PDX models may not be predictive of patient responses in the context of drugs which target tumor metabolism (Lane et al. 2016).

PDXs are not suitable for high throughput screening, or genetic manipulation (Gao and Chen 2015), but are extremely useful in situations where primary tumor material is scarce or rare, allowing long-term culture of much more diverse and heterogeneous tumor samples (Zhang et al. 2013). They can be developed from treatment resistant or recurrent tumor tissue (Aparicio et al. 2015; Townsend et al. 2016; Stewart et al. 2017), allowing the mechanisms involved to be explored. Various depositories of diverse treated/untreated tumor PDXs have been produced or are being developed, (EuroPDX consortium, the Public Repository of Xenografts, and the National Cancer Institute Patient-derived Models Repository) some of which are open source, such as that provided by Stewart et al., encompassing pediatric solid tumors, from 168 patients and 12 cancer types (Klco et al. 2014; Gao et al. 2015; Stewart et al. 2017; Townsend et al. 2016; Bruna et al. 2016).

d) Improving animal models

Animal models have been improved by 'humanizing' some aspects of the host, introducing human stromal elements or immune cells and thus humanizing the TME to some extent and allowing new immunotherapies to be explored (Proia, 2006; Vudattu et al. 2014; Morton et al. 2016). Such methods have been used to engineer the primary tumor environment, or mimic metastatic niches in breast and prostate cancers (Wang et al. 2010; Thibaudeau et al. 2014; Hesami et al. 2014; Holzapfel et al. 2014). One group has reported humanizing immunocompromised mice using aspirated hematopoietic stem and progenitor cells from bone marrow biopsies taken from cancer patients (Werner-Klein et al. 2014). However, the main obstacles to the

use of animal models remain high cost and low throughput. The pros and cons of these animal models are outlined in Table 1.

3D *in vitro* models

Xenograft models can have long latency periods; PDXs are maintained in a murine host and the availability and access to fresh human tumor tissue is a limiting factor for many researchers. Further, the trend in ethical research is to try to limit the use of animals wherever possible. There is therefore, a strong need for low cost, high throughput, clinically and physiologically relevant models for cancer research. Several studies have illustrated that 3D gene signatures can predict prognosis in several independent datasets (Fournier et al. 2006; Martin et al. 2008), suggesting that 3D *in vitro* 'organoid' models may help bridge the gap between pre-clinical translation and clinical trials.

Cancer stem cells (CSCs) form a subpopulation of cancer cells within a tumor that have been attributed with the ability to initiate primary, metastatic and recurrent tumor growth, and to be involved in treatment resistance (Mitra et al. 2015; Bomken et al. 2010). These cells have become an obvious focus for anti-cancer research. Tumor spheroids and organoids (see below) are both enriched for populations of CSCs (Lancaster and Knoblich 2014; Ishiguro et al. 2017; Capodanno et al. 2018). This allows for fast expansion of both organoid and spheroid culture systems, once the initial population of these 3D structures has been established. Further, both of these models can be used to harvest CSCs for research purposes (Ishiguro et al. 2017; Wang et al. 2018); an improvement on the traditional methodology for isolation and enrichment of these cells. CSCs from other mammals, for example from canine cancers, can also be obtained from this approach aiding studies in comparative and veterinary oncology (Capodanno et al; 2018).

a) Spheroids

Many cancer cell lines can be grown as small avascular spheroids using 3D scaffolds, hydrogels/collagen gels, or suspension culture as illustrated in Figure1 (Frederich et al. 2009; Li and Lu 2011, Verjans et al. 2017). These generate a more

physiological model for cancer drug testing, displaying increased heterogeneity, cell-cell and cell-extracellular matrix (ECM) interactions, and differential gene/protein expression in comparison with monolayer cultures (Yamada and Cukierman 2007, Loessner et al. 2010). Drug and cell signaling responses differ when 3D and 2D methodologies are evaluated (dit Faute et al. 2002; Yamada and Cukierman, 2007; Loessner et al. 2010; Ravi et al. 2014). For example, in SKBR-3 breast cancer cells, the HER2 and HER3 receptors heterodimerise in 2D cultures, but HER2 forms homodimers in spheroids, with an increased response to HER2 inhibition (Pickl and Ries 2009). 3D cultures of human pancreatic, ovarian, liver and lung cancer cells exhibit increased chemoresistance in contrast to 2D culture via mechanisms that involve interactions with the ECM/TME (Sethi et al. 1999; Loessner et al. 2010; Longati et al. 2013; Ekert et al. 2014).

In solid tumors, rapid cell growth induces gradients of hypoxia and acidosis to develop, affecting sensitivity to radiation and chemotherapy (Gatenby et al. 2007; Correia and Bissell 2012; Ward et al. 2013). Although spheroids are grown under atmospheric oxygen tensions they develop O_2, CO_2, pH and metabolite gradients as they reach approximately 400 – 600 uM in size, modelling the biology of solid tumors as illustrated in Figure 1 a and b. (Minchinton and Tannock 2006; Poussegur et al. 2006; Friedrich et al. 2009). These 3D models are now a necessity, particularly since novel treatment approaches targeting both pHi regulation and/or glycolysis in hypoxic tumors are expected to be useful in combination with systemic and/or radiation therapy (Riffle et al. 2017). One reason for translational drug failure may be the lack of exploration of the effects of new drugs in conditions found in the TME.

Spheroids can be produced from cancer cells adapted by CRISPR, siRNA, shRNA or inducible transgenics and can incorporate other cell types such as fibroblasts or be embedded in ECM proteins to examine cell-cell and cell-matrix interactions, migration and invasion (Schmeichel and Bissell 2003; Heneweer et al. 2005; Hsiao et al. 2009; Ingram et al. 2010; Herrmann et al. 2014; McCracken et al. 2014; Ward et al. 2015) (Figure 2a). For example, the leading edge of pancreatic cancer cells contain RhoA-dependent projections which can be targeted by anti-metastatic treatments; these were first identified in 3D culture models (Timpson et

al. 2011). Tumor-derived human and murine breast cancer spheroids embedded into collagen, showed that cells expressing basal epithelial markers lead to collective invasion, both in 3D culture and in *in vivo* studies across breast cancer sub-types (Cheung et al. 2013).

Co-culture of spheroids and cancer-associated fibroblasts (CAFs) demonstrated that CAFs influence treatment resistance and invasion (Crawford et al., 2009; Li and Lu 2011; Horie et al. 2012; Straussman et al. 2012; Clark et al. 2013; Jaganathan et al. 2014; Kim et al. 2015). Co-culture systems have been used to replicate the metastatic niche of prostate cancer cells in bone using co-culture with osteoblasts and endothelial cells (Hsiao et al. 2009). Other 3D co-culture systems, using niches from lung or bone marrow stroma, demonstrated the role of TSP1 in tumor dormancy (Ghajar et al. 2013), while the relationship between brain endothelial cells and cancer stem cells have been examined in glioblastoma using 3D models of the perivascular niche (Infanger et al. 2013). A 3D co-culture of pancreatic stellate cells with PDA cells has produced a model that could be used to investigate PDA-stroma interactions in high throughput assays for drug screening (Ware et al. 2016). Spheroid generation using a defined size microwell assays with non-adherent hydrogels can generate uniform spheroids from cervical, breast, and head and neck squamous cells carcinoma cells within 3 to 6 days (Singh et al. 2015, 2016), making this system useful for high throughput assays.

b) Organoids

Organoids are created by culturing tissue samples with factors to stimulate the replication of pluripotent stem cells. It can take 3 weeks for organoids to grow to approximately 1 mm^3 at which stage they contain the major cell types of the original tissue (Lancaster and Knoblich, 2014). Tumor organoids can be developed from tumor tissue or from isolated CSCs, and replicate many of the features of the primary tumor. For example, glioblastoma organoids from patient-derived CSCs demonstrated heterogeneity, a proliferative outer rim surrounding a hypoxic interior of senescent cells with dispersed quiescent radioresistant CSCs. Tumors generated

from these patient-derived organoids, showed the histological features of the parental tumor (Hubert et al. 2016).

Organoids can also be developed from murine tumor models. These have been used to explore the role of β-catenin and PI3K in colorectal cancer; a screen of inhibitors using this system associated 4EPB1 and Akt with cellular survival and motility in disease progression (Riemer et al. 2017). They have been developed from resected tissue and biopsies from colorectal (CRC) and prostate cancer, breast cancer, PDA, liver cancer, metaplastic epithelia from Barrett's esophagus, and also from circulating tumor cells (Sato et al. 2011; Gao et al. 2014; Boj et al. 2015; Weeber et al. 2015; Broutier et al. 2017; Schutte et al. 2017, Sachs et al. 2018).

Tumor organoids contain differentiated cell types, are biologically stable, can be frozen for later use and expanded once established (Sato et al. 2011; Sachs and Clevers 2014). Organoids allow the culture of tumors from individual patients, while maintaining the genetic and morphological diversity found within the corresponding patient tumor (van de Wetering et al. 2015; Weeber et al. 2015; Broutier et al. 2017; Pauli et al. 2017); for example, CRC organoids displayed a high degree of genetic similarity with the tumor tissue of origin (Schutte et al. 2017). PDA organoids generated from different stages of the cancer accurately replicated tumor progression based on transcriptomic and proteomic analysis (Boj et al. 2015) and demonstrated little change in histology, morphology, cytology or expression of differentiation markers from the originating tumor after 16 days in culture (Huang et al. 2015). It remains to be established whether these similarities are sustained long term. Although several studies suggest that mutation patterns are maintained during multiple passages (van de Wetering et al. 2015; Weeber et al. 2015; Fujii et al. 2016; Pauli et al. 2017; Schutte et al. 2017), one study using fluorescent markers showed that one color pre-dominated after 30–40 days in culture, suggesting clonal drift over time (Fujii et al. 2016). Organoids can also provide tissue for the study of rare cancers or those where cell line models are scarce. A further advantage is that control organoids from the same patient, can be developed from tumor-adjacent tissue. Several groups have reported the culturing of matched normal and tumor organoids

from treatment naïve surgical resections or biopsies (Boj et al. 2015; Van de Wetering et al. 2015).

Organoid cultures still lack tumor stroma, vasculature and immune cell interactions and those derived from early stage cancers appear to grow more quickly; therefore more malignant tumors may need optimized conditions for growth. They will not mirror the accumulated genetic mutations found in later stages of progression if developed from early stage tumors, but drug treatments should allow generation of treatment resistant models. This methodology may lead to personalization of drug testing for a plethora of cancers (Sachs and Clevers 2014), but the length of time for growth can vary depending on the cancer type. Costs may be also an issue and it is still unclear whether this method is applicable to all epithelial cancers.

Some of these issues are overcome by marrying organoid and PDX methodology. In one PDA model, tumor organoids were grown in orthotopic sites in mice, where they formed early-grade tumors that could develop and progress from local invasion to metastasis, while maintaining the histology of the original tumor tissue (Boj et al. 2016). This methodology was used to characterize specific alterations between pre-malignant and malignant states using transcriptomic and proteomic studies. Organoids grown from normal tissue, can be genetically engineered, using technologies such as CRISPR and therefore manipulated to study specific cancer mutations and cancer development and metastasis (Dekkers et al. 2013; Schwank et al. 2013; Matano et al. 2015; Fujii et al. 2016; Drost et al. 2017; Roper et al. 2017). As with spheroids, other cell types can be cultured with organoids. For example, co-culture of intraepithelial lymphocytes with murine intestinal organoids, showed lymphocytes entering and leaving organoids (Nozaki et al. 2016), but better tumor models will be achieved with the co-culture of stromal elements, particularly in tumors such as PDA, where the stroma is particularly important.

Organoids have been extremely useful in the study of some types of cancer. For example, *de novo* prostate cancer cell line culture is difficult, with only seven widely available cell lines that lack expression of many of the genetic lesions involved in this cancer. Using organoids from prostate cancer patients showed that they developed similarities to the histology of the original tumor, and contained many of the

mutations in *SPOP*, and *FOXA1* as well as *CHD1* loss, which are known to be involved in prostate cancer (Gao et al. 2014). Further these organoids demonstrated differences in responses to drugs and after 3 months culture, the organoid lines were shown to still share matching somatic mutations and transcriptomes with the original tumor. Organoids have been also been established from rare CRC tumors including neuroendocrine cancers and serrated adenomas, and grown from biopsies from metastatic disease and from circulating tumor cells (Gao et al. 2014; Yu et al. 2014, Fujii et al. 2016).

CRC organoids responded to drug treatment based on the molecular status of the original tumor (Gao et al. 2014; Van de Wetering et al. 2015). Screening of 83 drugs in these organoids confirmed that *TP53* loss of function mutants did not respond to the MDM2 inhibitor nutlin 3a, nor *RAS* mutants to EGFR inhibitors and demonstrated responsiveness of *RNF43* mutants to inhibitors of Wnt secretion. Organoids generated from liver metastases were more metastatic in xenograft culture even though the gene expression and mutational status were found to be comparable with the original tumor (Van de Wetering et al. 2015). Comparisons between a primary gastric cancer and its ovarian metastatic growth, found that the metastasis lost amplification of the *FGFR2* gene, but had gained alteration in transforming growth factor-β receptor 2 (Nadauld et al. 2014). Other research illustrated that combination treatment using pan-HER and MEK inhibitors caused cell-cycle arrest in $Kras^{G12D}$ mutant CRC organoids, but cell death in *KRAS* wild-type organoids; combining this treatment with BCL-2 inhibition, surmounted this resistance, a result counter to those seen in cell lines (Verissimo et al. 2016). Screens of single compounds or co-treatments in CRC, endometrial and uterine cancer organoids have uncovered several possible original treatment strategies (Pauli et al. 2017).

A large multicentre, prospective trial (TUMOROID – NL49002.031.14) is assessing whether the drug responses of organoids can be used to evaluate treatment responses in metastatic CRC, breast or NSCLC patients. The use of organoids in patient selection for clinical trials is under examination in the SENSOR study (NL50400.031.14 EudraCT 2014-003811-13), using biopsies from metastatic CRC and NSCLC. This is of importance since it is likely that potential novel treatments

could fail if evaluated in an inappropriate patient group which is of real concern in the development of specific, targeted therapies. There are several initiatives to develop both cancer and normal organoids as resources for researchers. For example, the Hubrecht Organoid Technology (HUB) biobank, which forms a section of the Human Cancer Models Initiative, aims to generate around 1,000 models in collaboration with The National Cancer Research Institute, Cancer Research UK, and the Wellcome Trust Sanger Institute. This initiative develops and analyses tumor organoids from pancreatic, prostate, breast, CRC and lung tumors, collating patient data and drug sensitivity. It was recently reported that over 100 breast cancer organoids from primary and metastatic tumours have also been cultivated (Sachs et al. 2018).

c) Tumor Explants

Tumor explants are a type of organoid derived from small tumor fragments often cultured on a semi-solid support (Figure 2b). Using primary tumor tissue in *ex vivo* culture maintains the stromal components of the tumor and the tumor architecture (van der Kuip et al. 2006; Gu et al. 2013a; Witkiewicz et al. 2015; Karekla et al. 2017).

Cultured explants offer a route towards precision medicine because multiple drug responses can be identified and monitored within days (Ward et al. 2015), a far more acceptable time scale in comparison with PDXs or organoids. *Ex vivo* tumor explants using naive biopsy tissue could provide analogous insights to neoadjuvant clinical trials, to verify responsiveness of the primary tumor to specific therapies, and could be used to examine novel therapies, co-treatments, and treatment scheduling. Explants can survive for over a month in culture, (Leeper et al. 2011; 2012; Katz et al. 2012; Ward et al. 2015); they reflect the heterogeneity of the tumor structure, are a good model to target microenvironmental adaptations and can allow comprehensive testing of pre-clinical models in heterogeneous tumor material subtypes. If tumor growth recurs, post-treatment resistance in individual patients can be investigated if tumor tissue is available. One of the difficulties in modelling breast cancer is the heterogeneity of the disease as observed in the clinic, since

breast cancer subtypes often show differential responses to therapies. *Ex vivo* culture of unselected breast tumor explants can overcome this difficulty. Studies show that explanted breast tumor tissue conserved ER, PR and HER2 status and proliferation rates in comparison with the original primary tumor tissue, which demonstrates the preservation of many characteristics of the original tumor in the *ex vivo* model (Dean et al. 2012). Tumor explants have also been used to examine novel carbonic anhydrase inhibitors in breast and topical chemotherapy in urothelial carcinoma (Bolenz et al. 2009; Ward et al. 2015).

Studies using human breast tumor explants illustrated that inhibitors of Rac GTPases (EHT 1864), and STAT3 (Stattic) could prevent spread of breast cancers and cell proliferation at the invading edge of a tumor (Katz et al. 2012). In other work, treatment of breast cancer explants with the CDK4/6 inhibitor, PD-0332991 showed suppression of proliferation as measured by Ki67 staining, in all but a small subset of explants resistant to treatment. In these resistant tumors, lymph node metastases were also unaffected by treatment, and further research demonstrated that this was linked to the lack of the retinoblastoma (RB) tumor suppressor protein (Dean et al. 2012).

PD-0332991 has also been examined in PDA tumor explants (Witkiewicz et al. 2015). The CDK4/6 inhibitor, p16ink4a is commonly lost in PDA, but in most models and in xenografts, inhibition of CDK4/6 is a fairly ineffective treatment and resistance quickly develops. In the explant model, however, CDK4/6 inhibition suppressed proliferation in all explants tested, except for one demonstrating loss of RB; a result mirrored in PDX of the same tumors, modelled in parallel (Witkiewicz et al. 2015), suggesting differential drug responses across systems. A study in prostate cancer explants using the Jak2 inhibitor AZD 1480 (Gu et al. 2013b), demonstrated that responsive explants underwent apoptosis when nuclear Stat5a/b decreased in response to the inhibitor, while in resistant explants, it did not. Prostate tumor explants have also been used to test the effectiveness of novel Stat5a/b inhibitory compounds (Liao et al. 2015)

Explants have been used to examine the links between metabolism, hypoxia and inflammation in Barrett esophagus, a preneoplastic lesion which can progress to

esophageal adenocarcinoma (Phelan et al. 2016), and to examine the role of bile acids in gastroduodenal reflux which causes increased expression of MUC1, associated with early carcinogenic changes in this condition (Mariette et al. 2008). They have also been used in head and neck cancer to study the effectiveness of a phytochemical treatment, Lupeol (Bhattacharyya et al. 2017). Results showed a good response to Lupeol, and using explants from a cisplatin-resistant tumor, it was found that this molecule could resensitize the tumors to cisplatin (Bhattacharyya et al. 2017). In non-small cell lung carcinoma, tumor explants could be used to predict patient response to therapy and to monitor clinically relevant biomarkers (Karekla et al. 2017). Explants have been used to confirm and investigate changes between low and high grade tumors in several settings such as endometrial cancer (Cornel et al. 2012). Analysis of sarcoma development suggests that while the p53 tumor suppressor pathway may be intact, the p53 ubiquitin ligase, *MDM2*, is often amplified or overexpressed (Toledo and Wahl 2006). Although pre-clinical studies in cell lines with amplification of *MDM2* using the MDM2 inhibitor Nutlin-3a produced positive results, only 1 patient in 20 showed a partial response in clinical trials in sarcoma patients (Muller et al. 2007; Ray-Coquard et al. 2012). Pishas et al. used sarcoma tumor explants from a range of subtypes and showed that there was no correlation between *MDM2* amplification and response to Nutlin-3a (Pishas et al. 2013) and that non-responsiveness to the drug was conferred by hypermethylation of the p53 target gene *GADD45A*. Explants have also been used in radiation studies. For example, the effectiveness of inhibiting TGF-β as a treatment or possible radiosensitiser of high grade glioma has been examined in tumor explants (Bayin et al. 2016). Results showed that most, but not all high grade gliomas, were responsive, and the authors suggest, that tumor explants could provide a rapid platform to determine patient response to this therapy and stratify treatment.

Currently, the main *in vivo* methodology for studying metastasis, generally involves the use of mouse models. Culture of tumor explants enables invasive changes in an appropriate TME to be assessed and novel therapeutics to be analyzed in patient samples in a fast and quantitative format (Ward et al. 2015) (Figure 2b). The effect of different ECM components and stromal cells can also be evaluated and

monitored in this system. The main benefits of explants in these assays are that growth/response/invasion can be monitored continuously and appraised at any time over the course of an experiment and after use the explant tissue can be fixed and analyzed.

Normal skin explants are being used to investigate tumor immunology and possible anti-tumor vaccination development. One strategy currently used is to load dendritic cells with tumor associated antigens, but the costs of generating these cells and treating them is extremely high. The use of skin tissue explants to load resident dendritic cells with tumor antigens *in situ*, has been successfully explored as an alternative method (Ruben et al. 2014). Immune checkpoint inhibition has demonstrated promise in several malignancies, but successful treatment is dependent on infiltration of $CD8^+$ T cells. Prostate tumor explants were successfully treated to increase numbers of $CD8^+$ T cells while decreasing numbers of immune suppressor cells, (Muthuswamy et al. 2016), illustrating their use in investigation of immunotherapeutic strategies. At present, one drawback of tumor explants is the lack of vascularization which prevents study of angiogenesis and its inhibitors. However, novel research into vascularization of tumor explants has been recently published (Bazou et al. 2016). This group has developed a method of inducing vasculature formation into xenografts of human breast cancer and melanoma, *ex vivo*, and consider that it should be possible to adapt this methodology for vascularization of human tumor explants. The advantages and disadvantages of 3D *in vitro* organoid systems are listed in Table 2.

Conclusions

It has been estimated that over 90% of drugs that make it through to clinical trials fail because of lack of efficacy and concerns over safety (Townsend et al. 2016, Ledford, 2011). Given the particularly high attrition rate of novel anti-cancer therapies during progression from pre-clinical to clinical trials, it is obvious that current testing methodologies are problematic. Better, more physiological systems are clearly needed for a) drug testing and b) patient participation in an era of more targeted treatments and personalized medicine. The ‘ideal’ tumor model would be able to mimic the development of malignant cells, angiogenesis, stromal development,

immune responses, metastasis, therapeutic efficacy, and progression to resistant disease. This chapter shows that there is not one specific methodology that can fulfil these criteria. Therefore more than one model system will be required to provide better translational results.

Cell lines are a suitable model for drug screening, but lead compounds need to be tested in more intricate animal models, that allow for pharmacokinetic and toxicity studies that can monitor off target effects. These *in vivo* models are useful, and GEMMs and PDXs can reflect many of the features of the tumor from which they were developed. But the time taken for tumor growth is not compatible with use as a clinical decision making tool in precision medicine. However, *in vitro* models specific to an individual patient (such as organoids or tumor explants), can be used to test drugs and guide treatment in a more specialized manner. Results from these systems are available within days and could thus prevent the use of ineffective drugs for a given patient and limit access to more expensive drugs to those likely to benefit.

Access to human tumor tissue is problematic for many scientists, but because organoids can be stored they can be used as a research resource and passed to other researchers. Organoids could also be developed from GEMM mouse models and used in cancer research. They would be particularly useful in the study of the preclinical stages of cancer, and in the study of cancer resurgence from residual disease. This methodology has been used to study recurrence in breast cancer, and showed that these residual cells develop to become transcriptionally divergent from both the original tumor and normal epithelium (Havas et al. 2017). Organoids can be used for high throughput drug screening assays that meet industry standards (Huang et al. 2015; Van de Wetering et al. 2015; Boehnke et al. 2016).

The most significant factor that is likely to govern the rate of success in oncology clinical trials, is matching the experimental models to the clinical malignancy. The pros and cons of experimental systems suggest that new therapeutics should be examined using several methods.

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Table 1

	Strengths	Weaknesses
Mouse Xenografts	- allow *in vivo* monitoring of tumor growth, drug toxicity and efficacy - reflect *in vivo* pH and oxygen gradients	- limited metastatic models - compromised immune responses - murine stromal elements
GEMMs	- share the genetic heterogeneity and histopathology of human tumors - replicate both the TME and stroma more accurately than xenografts	- model is still murine
PDXs	- pathology, growth and metastatic outcomes closely correspond to the original tumor - preserve the heterogeneity of different cancers - useful in cases where primary material is rare	- availability/access to fresh tumor tissue is a limiting factor - passaging is time consuming - stromal tumor elements lost over time - rate of engraftment is poor for some cancer types - deficiency in immune response

Table 1
Listing the advantages and disadvantages of *in vivo* tumor models.

Table 2

	Set-up Methods	Strengths	Weaknesses
Spheroids	Spheroids are set up using cancer cell lines from 2D culture. (various methods are illustrated in Figure 1). These can be grown from genetically modified cells and can incorporate other cell types	- exhibit cell/cell and cell/ECM interactions - mimic *in vivo* oxygen, pH and metabolic gradients - useful in high-throughput drug screening assays	- use cancer cell lines, which can lose the biological traits of the cancer from which they derived
Organoids	Organoids are developed from tissue samples/cancer stem cells and contain the cell types found in the tissue of origin. These cells can be genetically modified, and cultured with other cell types	- biologically stable - can be frozen for later use - allow the culture of tumors from individual patients - can provide tissues for the study of rare cancers or where cell line models are scarce - useful in high-throughput drug screening assay	- clonal drift may occur over time - high costs - lack tumor stroma, vasculature and immune cell interactions
Explants	Explants are derived from human-tumor tissue – usually biopsies. Genetic modification is difficult, but they can be cultured with other cell types	- maintain the stromal components and tissue architecture of the primary tumor - good model to target microenvironmental adaptations - maintain histology and proliferation rates of the original tumor - growth/response/invasion can be monitored continuously	- lack of vascularization prevents the study of angiogenesis and its inhibition

Table 2
Listing the advantages and disadvantages of 3D *in vitro* tumor models.

Figure Legends

Figure 1. Illustrating the physiological features of spheroids and several of the methods used to culture them. a) a schematic diagram of the different layers of a spheroid culture b) shows immunohistochemical staining for carbonic anhydrase IX, which is upregulated by hypoxia and indicates the hypoxic region c) spinner flask spheroid culture d) alginate culture e) hanging drop method.

Figure 2. Illustrating 3D invasion assays using human breast cancer cell spheroids and human breast cancer tumor explants. a) spheroids grown in spinner flasks and embedded in collagen I. Images were obtained at 0 h and 96h of culture. b) tumor explants prepared by trimming fat from biopsy samples and cutting into pieces approx. 1mm^3, before embedding in collagen I. Images were obtained at 0h and 120h of culture. Images were acquired using phase contrast microscopy. Original magnification x 25.